Kongeriget Danmark

Patent application No.: 0352/96

Date of filing: 27 Mar 1996

Applicants: GSF-Forschungszentrum fuer Umvelt und Gesundheit GmbH, Ingolstaedter Landstras se 1, Neuherberg, D-85758 Oberschleisshe im, DE; Bavarian Nordic Research Institute A/S, Smedeland 26B, DK-2600 Glostrup, DK

This is to certify the correctness of the following information:

The attached photocopy is a true copy of the following document:

- The specification, claims and abstract as filed with the application on the filing date indicated above.

Patentdirektoratet · Kongeriget Danmark ·

Erhvervsministeriet
Patentdirektoratet

TAASTRUP 31 Jul 1998

Karin Schlichting
Kontorfuldmægtig

BN 10 DK **27 Marts 1995**

Titel: Retrovirale vectorer og deres anvendelse.

Ansøgere: GSF-Forschungszentrum für Umwelt und Gesundheit GmbH
Ingolstädter Landstrasse 1, Neuherberg
D-85758 Oberschleissheim
Tyskland

og

Bavarian Nordic Research Institute A/S
Smedeland 26B,
DK-2600 Glostrup
Danmark

Opfindere:

Cytochrome P450 transducing retroviral vectors

The present invention relates to the transfer of a gene encoding a product that converts a harmless prodrug into active metabolites. It isoptionally included in a retroviral vector which undergoes promoter conversion (ProCon vector). The product of the inserted gene shows cytotoxicity by the metabolisation of cancer prodrugs. This strategy is useful for gene therapeutical treatment of a variety of tumors.

Background of the Invention

A variety of malignant tumors do not respond well to chemotherapy. The anti-cancer drugs used to treat tumors are in most cases applied systemically and therefore spread through the whole body of the patient. The high systemic dose of such drugs required for cancer treatment often is combined with unpleasant side-effects for the patient. In attempts to circumvent these problems cancer-prodrugs have been used. These have to be metabolized in the body before they become cytotoxic. Unfortunately, human tumors that contain appropriate high levels of the activating enzymes are rare. One of the main sites for activation of prodrugs is the liver. As a result the highest concentrations of the activated drug are found in this organ. To ensure that the tumor, at a distant site, receives a sufficent dose of the activated drug to be therapeutically effective, the concentration produced in the liver has to be raised commensurately. Again this leads to toxic side effects for the patient. One strategy by which these problems of high systemic concentration of activated drugs could be circumvented is by the activation of the drug directly in or near the tumor. This could be achieved by introduction of the gene encoding the activating enzyme in an appropriate expression cassette, either into tumor cells or into nearby cells. Retroviral vectors are ideally suited for the stable delivery of therapeutic genes to cells since the retrovirus is able to integrate the DNA form of its genome into the host cell. Thus all daughter cells of an infected cell will carry the retroviral vector carrying the therapeutic gene. Retroviral vectors are the most commonly used gene transfer vehicles for the clinical trials that have been undertaken to date. Most of these trials have, however, taken an *ex vivo* approach where patient's cells have been isolated, modified in culture and then reintroduced into the patient.

It would be feasible to take patient's cells (either tumorigenic or normal), introduce the gene cassette encoding the activating enzymatic activity into them *in vitro*,

and then return them to the patient in the vicinity of the tumor. Such an autologous approach, however, suffers from the problem of the fact that for each patient cells must be cultured, transduced with the gene construct and successfully returned without infection by adventitious agents. The cost and time involved in such an approach limits the usefulness severely. Alternatively an allogenic approach could be envisaged, where cells are prepared that carry the appropriate gene cassette, and these standard cells are used for many different patients. Such an approach is much more feasible, assuming that problems of immune rejection can be overcome.
Ideally, however, the gene cassette encoding the activating enzyme should be introduced directly into the tumor cells or cells in its vicinity *in vivo* in the patient.

Since most tumors are not suitable for *ex vivo* gene therapy, the delivery of genes *in vivo* of course, introduces a variety of new problems. First of all, and above all, safety considerations have to be addressed.

A major concern for eventual *in vivo* gene therapy, both from a safety stand point and from a purely practical stand point, is the targeting of RVs. It is clear that therapeutic genes carried by vectors should not be indiscriminately expressed in all tissues and cells, but rather only in the requisite target cell. This is especially important if the genes to be transferred are such prodrug activating genes designed to ablate specific tumour cells. Ablation of other, nontarget cells would obviously be very undesirable. Targeting of the expression of carried prodrug activating genes may be achieved by ProCon vectors.

The essentially random integration of the proviral form of the retroviral genome into the genome of non-tumorigenic infected cells may lead to new genomic arrangments. The possibility that proto-oncogenes are activated by viral insertion in the genome of non-tumorgenic cells and thereby may cause new cancer has posed an ethical problem. Most researchers would agree that the probability of a replication defective retrovirus (RV), such as all those currently used, integrating into or near a cellular gene involving in controlling cell proliferation is vanishingly small. However it is generally also assumed that the explosive expansion of a population of replication competent retroviruses from a single infection event, will eventually provide enough integration events to make such a phenotypic integration a very real possibility.

Retroviral vector systems are optimized to minimize the chance of replication competent virus being present. However it has been well documented that recombination events between components of the retroviral vector system can lead to the generation of potentially pathogenic replication competent virus and a number of generations of vector systems have been constructed to minimize this risk of recombination (reviewed in Salmons and Günzburg, 1993).

Retroviral vector systems consist of two components:

1) the retroviral vector itself is a modified retrovirus (vector plasmid) in which the genes encoding for the viral proteins have been replaced by therapeutic genes. Since the replacement of the genes encoding for the viral proteins effectively cripples the virus it must be rescued by the second component in the system which provides the missing viral proteins to the modified retrovirus.

The second component is:

2) a cell line that produces large quantities of the viral proteins, however lacks the ability to produce replication competent virus. This cell line is known as the packaging cell line and consists of a cell line transfected with a second plasmid carrying the genes enabling the modified retroviral vector to be packaged.

To generate the packaged vector, the vector plasmid is transfected into the packaging cell line. Under these conditions the modified retroviral genome including the inserted therapeutic gene is transcribed from the vector plasmid and packaged into the modified retroviral particles (recombinant viral particles). This recombinant virus is then used to infect tumor cells in which the vector genome and any cytotoxic gene becomes integrated into the target cell's DNA. A cell infected with such a recombinant viral particle cannot produce new vector virus since no viral proteins are present in these cells. However the DNA of the vector carrying the therapeutic is integrated in the cell's DNA and can now be expressed in the infected cell.

A variety of cytotoxic genes carried by retroviral vectors have already been tested. These genes encode enzymes which convert substances that are pharmacodynamically and toxicologically inert even at high dose-levels but which can be converted *in vivo* to highly active species (Connors, 1995). The

conversion from nontoxic and inactive prodrug to an active from can be the result of an altered pH at the site of activation or the result of changes in temperature or salt concentration. However, in most cases, tissue specific activation of a prodrug is the result of metabolism by an enzyme that is either unique for the tissue, or present at a higher concentration (compared with other tissues) so that it activates the prodrug far more efficiently. Prodrugs have been used in many areas of medicine for more than 30 years and there is a considerable body of experience in their design for use in different circumstances (Bondegaard, 1985).

In cancer chemotherapy appropriately designed prodrugs have been found to be effective in the treatment of animal tumors possessing high levels of an activating enzyme (Connors, 1966, Cobb, 1969). Clinical results were, however, disappointing since it was found that human cancers that contained appropriately high levels of activating enzymes were rare (Connors, 1986). Virally directed enzyme prodrug therapy (VDEPT) and the more general gene directed enzyme prodrug therapy (GDEPT) are related in that they also aim to destroy tumor cells by the tumor specific activation of a prodrug. However, in this case, the gene encoding the enzyme is either specifically targeted to malignant cells or is under the control of a specific promoter.

Up to now most of the efforts directed towards prodrug therapy have concentrated on the use of the human Herpes Simplex Virus thymidine kinase (HSV-tk) as a suicide gene. Although the HSV-tk enzyme in combination with the prodrug ganciclovir (GCV) has been recommended as a good system for GDEPT (Culver et al., 1992; Ram et al., 1993; Chen et al., 1994) there are a number of theoretical considerations that would suggest that it is by no means the best combination. First, it is an S-phase specific agent with no effect on resting cells. This is because the GCV monophosphate is short lived and has to be present when cells are entering the S-phase to give a toxic effect. The HSV-tk phosphorylates GCV to the monophsophate form (a reaction that cannot be performed by mammalian enzymes) which is then phosphorylated by cellular enzymes to the triphosphate form and incorporated into DNA. Second, the active drug is a triphosphate and would not be expected to diffuse freely to cause a bystander effect. However a bystander effect has been observed both *in vitro* and *in vivo* although metabolic cooperation appears to be involved and in the latter case some of the effect may be an indirect one involving an immune component (Bi et al., 1993, Vile et al., 1993, Freeman et al., 1993). One disadvantage is that the bystander effect is dependent on a cell-cell contact. This may be due to the

presence of gap-junctions formed by intimate contact between the transduced and the surrounding cells which enable the transfer of phosphorylated ganciclovir.

Recently, interesting results have been reported with cells that have been transfected with the gene encoding the rat cytochrome P450 form 2B1 and then treated with cyclophosphamide (Chen et al., 1994).

Cytochrome P450's form a broad group of mono-oxygenases that catalyze oxidation of a wide range of substrates. They are produced by some bacteria, yeasts, and by higher organisms, where they play a role in detoxification of xenobiotics, bioactivation reactions, and metabolism of various endogenous compounds.

Cytochrome P450 catalyses the hydroxylation of the commonly used cancer prodrugs cyclophosphamide (CPA) and ifosfamide to their active toxic forms. Normally the expression of the patient's endogenous cytochrome P450 gene is limited to the liver, and anti-tumor effects of systemically applied CPAs depend upon the subsequent systemic distribution of toxic drug metabolites from the liver. This has led to toxicity problems since the activated drug not only affects the tumor but also affects other normal patient tissues such as bone marrow and kidney.

A therapeutic approach, where the cytochrome P450 gene is selectively introduced directly into tumor cells, and overexpressed in these cells, would circumvent this problem. Toxic metabolites produced from the transduced tumor cells affect surrounding non-transduced tumor cells in a concentration gradient dependent manner. An additional advantage of the cytochrome P-450/CPA system is the lack of dependency upon cell replication for cytotoxic effects on the surrounding cells. This is because one of the active metabolites generated causes interstrand crosslinks regardless of the cell cycle phase. Later on, during DNA synthesis, these interstrand crosslinks result in cell death.

A number of retroviral vector systems have been previously described that should allow targeting of the carried cytotoxic genes (Salmons and Gunzburg, 1993). Most of these approaches involve either limiting the infection event to predefined cell types or using heterologous promoters to direct expression of linked heterologous therapeutic genes to specific tumor cell types. Heterologous promoters are used which should drive expression of linked genes only in the cell

type in which this promoter is normally active or/and additionally controllable. These promoters have previously been inserted, in combination with the therapeutic gene, in the body of the retroviral vectors, in place of the *gag, pol* or *env* genes.

The retroviral Long Terminal Repeat (LTR) flanking these genes carries the retroviral promoter, which is generally non-specific in that it can drive expression in many different cell types (Majors, 1990). Promoter interference between the LTR promoter, and heterologous internal promoters, such as the tissue specific promoters, described above, has been reported. Additionally, it is known that retroviral LTRs harbor strong enhancers that can, either independently, or in conjunction with the retroviral promoter, influence expression of cellular genes near the site of integration of the retrovirus. This mechanism has been shown to contribute to tumorigenicity in animals (van Lohuizen and Berns, 1990). These two observations have encouraged the development of Self-Inactivating-Vectors (SIN) in which retroviral promoters are functionally inactivated in the target cell (WO94/29437). Further modifications of these vectors include the insertion of promoter gene cassettes within the LTR region to create double copy vectors (WO 89/11539). However, in both these vectors the heterologous promoters inserted either in the body of the vector, or in the LTR region are directly linked to the therapeutic gene.

The previously described SIN vector mentioned above carrying a deleted 3´LTR (WO94/29437) utilizes in addition a heterologous promoter such as that of Cytomegalovirus (CMV), instead of the retroviral 5´LTR promoter (U3-free 5´LTR) to drive expression of the vector construct in the packaging cell line. A heterologous polyadenylation signal is also included in the 3´LTR (WO94/29437).

The object of the present invention is the construction of a safe retroviral vector, which harbours a cytochrome P450 gene as a therapeutic principle. This novel vector carries heterologous constitutive, inducible or tissue specific promoter and/or regulatory elements in the 3´LTR which, after infection become duplicated and translocated to the 5´LTR in the target cell. Thus in the infected cell the introduced promoter controls the expression of the cytochrome P450 gene, which is inserted into the body of the vector. This vector does not undergo self-inactivation - but instead promoter exchange, giving rise to the name ProCon vector for Promoter Conversion vectors.

Since Promoter Conversion does not result in Self-Inactivation, the retroviral vector will be transcriptionally active in the target cell. Additionally both LTRs will consist to a large extent of heterologous promoter/enhancer sequences in the target cell. This will reduce the likelihood of the integrated vector in the target cell being subject to the same inactivation over long periods as has been described for conventional vectors (Xu ***et al.***, 1989) and also will reduce the chance of recombination with endogenous retroviral sequences to generate potentially pathogenic replication competent virus, increasing the safety of the system.

According to the invention the 5´LTR of the retroviral vector construct is not modified, and expression of the viral vector in the packaging cells is driven by the normal retroviral U3 promoter. Normal retroviral polyadenylation is allowed, and no heterologous polyadenylation signals are included in the 3´LTR. This is important for the development of *in vivo* gene therapy strategies, since the normal physiological regulation of the virus, through the normal viral promoter, and possibly also involving the normal viral control of polyadenylation, will prevail over long periods *in vivo* whilst the packaging cells are producing recombinant virus.

To achieve the foregoing and other objects, the invention provides a retroviral vector undergoing promoter conversion comprising a 5' LTR region of the structure U3-R-U5; one or more coding sequences selected from the group of genes known as cytochrome P450 genes; and a 3' LTR region comprising a completely or partially deleted U3 region wherein said deleted U3 region is replaced by a heterologous promoter, followed by the R and U5 region.

Said promoter can either be constitutive as the Cytomegalovirus (CMV) immediate early promotor/enhancer, inducible such as by glucocorticoid hormones (eg the MMTV promoter) or target cell specific.

The target cell specific regulatory elements and promoters are selected from one or more elements of any gene but in this embodiment may be from promoters including carbonic anhydrase II and ß-glucokinase regulatory elements and promoters, lymphocyte specific regulatory elements and promoters including carbonic anhydrase II and ß-glucokinase regulatory elements and promoters, lymphocyte specific regulatory elements of Whey Acidic Protein (WAP), Mouse Mammary Tumour Virus (MMTV), ß-lactoglobulin and casein specific regulatory elements and promoters, pancreas specific regulatory elements and promoters including immunoglobulin and MMTV lymphocytic specific regulatory elements

and promoters and MMTV specific regulatory elements and promoters conferring responsiveness to glucocorticoid hormones or directing expression to the mammary gland. Other promoters include for example the CD4, CD34, and IL2 promoters. Said regulatory elements and promoters regulate preferably the expression of said retroviral vector.
The LTR regions are selected from at least one element of the group consisting of LTRs of Murine Leukaemia Virus (MLV), Mouse Mammary Tumour Virus (MMTV), Murine Sarcoma Virus (MSV), Simian Immunodeficiency Virus (SIV), Human Immunodeficiency Virus (HIV), Human T-cell Leukaemia Virus (HTLV), Feline Immunodeficiency Virus (FIV), Feline Leukaemia Virus (FELV), Bovine Leukaemia Virus (BLV) and Mason-Pfizer-Monkey virus (MPMV).

The retroviral vector is based on a LXSN vector (Miller and Rosman, 1989), pBAG (Price et al. 1987) or a hybrid of both.
The coding sequence of the therapeutic gene may be any cytochrome P450 gene but most preferably is the rat cytochrome P450 form 2B1 defined by Fuji-Kuriyama et al., 1982.

In a further embodiment of the invention a retroviral vector system is provided comprising a retroviral vector as described above as a first component and a packaging cell line harbouring at least one retroviral or recombinant retroviral construct coding for proteins required for said retroviral vector to be packaged.

The packaging cell line harbours retroviral or recombinant retroviral constructs coding for those retroviral proteins which are not encoded in said retroviral vector. The packaging cell line is preferably selected from an element of the group consisting of Ψ-2, Ψ -Crypt, Ψ-AM, GP+E-86, PA317 and GP+envAM-12, or of any of these supertransfected with recombinant constructs allowing expression of surface proteins from other enveloped viruses.

The invention includes also mRNA resulting from a retroviral vector according to the invention.

According to the invention a retroviral vector is constructed in which the right-hand U3 region is altered, but the normal left-hand U3 structure is maintained; the vector can be normally transcribed into RNA utilizing the normal retroviral promoter located within the left-hand U3 region. However the generated RNA will only contain the altered right-hand U3 structure. In the infected target cell, after

reverse transcription, this altered U3 structure will be placed at both ends of the retroviral structure.

Gene expression is regulated by promoters. In the absence of promoter function a gene will not be expressed. The normal MLV retroviral promoter is fairly unselective in that it is active in most cell types (Majors, 1990). However a number of promoters exist that are either inducible or show activity only in very specific cell types. Such tissue-specific promoters will be ideal candidates for the regulation of P450 expression in retroviral vectors, limiting expression of the therapeutic genes to specific target cells.

In the packaging cell line the expression of the retroviral vector is regulated by the normal unselective retroviral promoter contained in the U3 region. However as soon as the vector enters the target cell promoter conversion occurs, and the P450 gene is expressed from a tissue specific or inducible promoter of choice inserted into the ProCon vector. Not only can virtually any tissue specific promoter be included in the system, providing for the selective targeting of a wide variety of different cell types, but additionally, following the conversion event, the structure and properties of the retroviral vector no longer resembles that of a virus. This, of course, has extremely important consequences from a safety point of view, since ordinary or state of the art retroviral vectors readily undergo genetic recombination with the retroviral packaging construct and/or endogenous retroviruses to produce potentially pathogenic viruses. Promoter conversion (ProCon) vectors do not resemble retroviruses because they no longer carry U3 retroviral promoters after conversion thus reducing the possibility of genetic recombination.

These vector systems will be used to generate recombinant virus that can be used to infect tumor or normal cells either *in vitro* or *in vivo*. In one embodiment of the invention the packaging cells will be enclosed in capsules. For an effective treatment, the virus producing cells have to survive long periods in the target organ after implantation and virus must be produced during this period and released from the packaging cells. Thereby, the packaging cells producing the virus would, in effect, constitute a small virus producing factory, placed at the site of application. This will allow efficient delivery of the recombinant virus *in vivo*. Alternatively infected normal cells, either of human origin, or those originating from other species will be encapsulated and implanted, providing a small prodrug conversion factory that can be sited near or in the tumor mass.

The long term effectivity of this approach depends on (1) protection of the cells from the host immune system, which would normally eliminate virus producing or infected cells, especially if the cells are from a different species as is usually the case for retroviral vector producing cells and (2) survival of the cells *in situ* for extended periods, which may require vascularisation.

It has been found that the continuous production of a vector virus from implanted packaging cells can be achieved by the appropriate encapsulation, in microcapsules with semipermeable membranes, of the virus producing packaging cells before implantation. Additionally, it has been found that the capsules according to the present invention become well engrafted in the host, become vascularized, and do not elicit a host immune or inflammatory response. These findings, together with the semipermeability of the capsule membrane, permits long term retroviral vector delivery *in vivo*.

An encapsulation technology providing for the encapsulation of virus producing packaging cells, and of virus infected or normal cells in a cellulose based material has been developed. Using this technique up to 10^{10}, but preferably 10^5-10^7 cells are encapsulated in electrolyte complex (e.g. from alginate and polylysine or, more preferably, cellulose sulphate and polydimethyldiallylammonium chloride) or other porous structures (such as polyamides, polysulphones). The resulting capsules can have a variable diameter between 0.01 and 5mm, but preferably are between 0.1 and 1mm. Consequently, capsules can be made to contain a variable number of cells. The capsule is semipermeable with pores that are large enough to allow viruses or prodrug molecules to pass through but small enough prevent cells of the immune system from accessing the cells thereby significantly reducing an immune response to these cells. The capsules and the encapsulated cells are cultivated in a normal cell culture medium (the nature of which depends on the cell line encapsulated) at standard conditions of humidity, temperature and CO_2 concentration.

After a suitable period in culture (normally not less than 1 hour and not exceeding 30 days), the cell containing capsules can be surgically implanted either directly, or by injection using a syringe into various areas. The capsules are well accepted by the host, and rapidly become vascularized. The pathology of sections made round the area of implantation show no evidence of inflammation or accumulation of cells indicating a cellular immune response.

At different times after the implantation of the encapsulated cells, the host can be treated with cyclosphosphamide or ifosfamide either locally or systemically. Cells infected with the cytochrome P450 expressing virus will convert these prodrugs to the active metabolites which cause alkylation and cross-linkage of DNA. Also cells carrying and expressing the cytochrome P450 gene (such as encapsulated infected cells, or encapsulated packaging cells) will also catalyse this conversion. In one embodiment of this invention these encapsulated infected or packaging cells will be either slowly dividing cells, or cells that have been treated with mitomycin C, low doses of radiation, or other means to prevent cell replication, and thus to prevent the cells from being themselves affected by the cytotoxic effects of the prodrugs.

Example 1

This example describes the construction of a retroviral expression vector for intratumoral infection which contains the gene for rat cytochrome P450 2B1.

Expression vector pLX2B1 was constructed by the combination of fragments obtained from plasmid pLX125 and the PCR product of rat-cytochrome-P450-2B1 gene.

The plasmid was first partially digested with the restriction enzyme XhoI to yield a vector which is linearized at position 3547. This linear plasmid was further digested with the restriction enzyme SspBI to remove a short fragment within the polylinker of pLX125. In a preparative gel the correctly cut vector fragment appeared as the largest band. Using the Quiaex protocoll, (Qiagen) the DNA in this band was eluted and purified from the gel.

To yield the rat cytochrome P450 2B1 gene cells of the rat hepatoma cell line HTC were lysed with solution D (4M guanidium thiocyanate, 25mM sodium citrate pH7, 0.5%N-laurylsarcosine sodium, 0.1M 2-mercaptoethanol) and total RNA extracted by adding 1/15 volume of 3M sodium acetate, in the same volume of watersaturated phenol and 1/5 volume of chloroform/isoamyalcolhol (49:1) were added and the whole mixture mixed vigorously. After 15 min on ice the extract was centrifuged 20min at 4°C at 10.000g. The RNA in the Aqueous phase was precipitated with one volume of isopropanol for 30min at -20°C and centrifuged at 10.000g at 4°C. The pellet was washed in 70% ethanol and left at room

temperature for 15 min. After 5 min centrifugation at 4°C and 10.000g the pellet was dried in a vacuum dryer and redissolved in 0.5% SDS solution.

The extracted RNA was reverse transcribed using the protocol for cDNA synthesis (Pharmacia). The resulting cDNA was used as template for a PCR. The primers were designed so that they contained a SspBI restriction site (underlined) in the lefthand primer (5'-AAGCCTGTACACTGGAGAGCATGCAC-3') and a XhoI site (undrlined) in the righthand primer (5'-CGATTACTCGAGACCTGGCTGCCTCA-3'). Both primers had additional bases at the 5'-end for higher efficiency of cleavage by the relevant restriction enzyme. The 1562 bp-product was digested with XhoI and SspBI to yield three fragments.

This longest fragment (1545 bp), containing the gene for cytochrome P450, was ligated into the XhoI/SspBI digested plasmid pLX125 to yield pLX2B1.

One day before lipofection $3x10^5$ retroviral packaging cells were seeded into 6cm petri or culture dishes. On the day of infection 2µg of pLX2B1 were mixed with 100µl serum free media. In parallel 15µl of Lipofectamine (Gibco BRL) was mixed with 100µl serumfree media. The plasmid containing solution was added to the Lipofectamine-mix and incubated for 45min. After 35 min the cell were washed once with 2ml serum free media. 800µl of serum free media were added to the lipofection-mix and the resulting 1ml was put onto the prepared cells. After 6 hours 1ml Dulbecco's modified Eagles medium containing 10% FCS was added. The next day the cells were trypsinized and 1:10 diluted and seeded on a 100mm dish. After 24h the media was replaced with medium containing the neomycin analog G418. Single cell clones or cell populations were isolated and analysed for expression of cytochrome P450.

Encapsulation of cells and implantation of encapsulated cells

The retroviral vector producing packaging cells are suspended in 1ml of 0.5-50%, but preferably 2-5%, anionic polymer (e.g. sodium cellulose sulphate) solution which also contains 5% fetal calf serum. This suspension is then dropped by a dispensing system (e.g. A-jet system or piezoelectric system) into a precipitation bath containing a stirred 0.5%-50% polymeric polycation (e.g. polydimethyldiallylammonium). The capsule formation occurs within milliseconds and the capsules containing cells are kept in the precipitation bath for 30 seconds

to 5 minuntes and then washed. The rapidity of this method ensures that the cells are not unduly stressed during the whole procedure (Stange et al., 1993).
Capsules containing NIH3T3 cells are introduced by "key hole" surgery near or in either transplanted or spontaneous tumors of BALB/c mice. About six capsules of 1mm diameter are inserted at each operation site. The site of surgery is closed by 1 suture. The mice are then treated with cyclophosphamide or ifosphamide locally, by direct intratumoral injection of 100µl of 20mg/ml or systemic concentrations of 130mg CPA/kg body weight i.p. and 40-60mg IFO/kg body weight i.p. for up to a maximum of 10 weeks. During this period tumor size and macroscopic appearance is monitored daily. The mice are then, sacrificed, the tissue containing the inserted capsules and tumor removed, and histological sections for normal and electron microscopy prepared. These sections clearly show good engraftment of the capsules, vascularization, and no evidence of the presence of lymphocytes indicative of a cellular immune response. These sections also show no sign of cell death or necrosis within the capsule. In contrast the tumor showed necrosis and macroscopically there was a clear reduction in size over the test period.

Example 2

This example describes the construction of a stable cell line which expresses rat cytochrome P450 form 2B1 constitutively.

To yield the mRNA from the rat cytochrome P450 form 2B1, a four week old female rat was sacrified, the liver taken out and immidiately frozen in liquid nitrogen. The frozen liver was put into sterilized filtered GTC-buffer (6M guanidium isothiocyanate, 5mM sodium citrate, 0,1M 2-mercaptoethanol, 0,5% sodium N-laurylsarkosyl) and homogenized at room temperature. For RNA seperation the liver extract was put onto a cushion of cesiumchloride (5,7M Cesiumchloride, 0,1M EDTA) and centrifuged in a swing-out rotor at 20°C and 32.000rpm over night. After complete removing the supernatant the pelleted RNA was redissolved in ice cold 10mM Tris pH 7,5 and preciptated overnight at -20°C with 1/15 volume 3M sodium acetate and 2,5 volumes ethanol. The RNA was spinned down 40min at 8000rpm and 4°C and the dried pellet resuspended in sterile water.

The extracted RNA was reverse transcribed using the protocol for cDNA synthesis (Pharmacia). The resulting cDNA was used as a template for the following PCR. The primer were so designed that they contained a EcoRI restriction site in the lefthand primer (5'-CGTGCGGAATTCGGCGGATTCAGCAT-3') and a EcoRVI site in the righthand primer (5'-ATAACGGATATCACCTGGCTGCCTCA-3'). Both primers had additional bases at the 5'-end for higher efficiency of the cutting enzyme. The 1588 bp-amplificate was digested with EcoRI and EcoRV to yield three fragments.

This longest fragment (1572 bp), containing the gene for cytochrome P450 2B1, was ligated to the EcoRI/EcoRV digested plasmid pcDNA3 (Invitrogen) to yield pc3/2B1.

Before the day of infection $3x10^5$ NIH3T3 cells were seeded into 35mm dishes. On the day of infection 2µg of pc3/2B1 was mixed with 100µl serum free media. In parallel 15µl Lipofectamine was mixed with 100µl serumfree media. The plasmid containing solution was added to the Lipofectamine-mix and incubated for 45min. After 35 min the cells were washed once with 2ml serum free media. 800µl of serum free media were added to the lipofection-mix and the resulting 1ml was put onto the prepared cells. After 6 hours 1ml DMEM (Glutamax) with 10% FCS were added.The next day the cells were trypsinized and diluted by factor ten and seeded on a 100mm dish. After 24h the media was replaced against neomycin media. After 14 days neomycin resistant clones were isolated and tested for presence and activity of the vector. Capsules containing these cells were produced as described in Example 1 and implanted in mice near the tumor site. After treatment with cyclophosphamide or ifosfamide the efficacy of treatment was evaluated as described above.

Claims:

1. A replication-defective retroviral vector carrying a cytochrome P450 gene under transcriptional control of target cell specific regulatory elements or promoters or X-ray inducible promoters.

2. A replication-defective retroviral vector according to claim 1, wherein the vector comprises a 5' LTR region of the structure U3-R-U5; one or more sequences selected from coding and non-coding sequences; and a 3' LTR region comprising a completely or partially deleted U3 region wherein said deleted U3 region is replaced by a polylinker sequence containing the target cell specific regulatory elements or promoters or an X-ray inducible promoter, followed by the U5 and R region, characterized in that at least one of the coding sequences is a cytochrome P450 gene.

3. A replication-defective retroviral vector according to claim 1-2, wherein the target cell specific regulatory element is the carbonic anhydrase II, WAP or MMTV regulatory sequences.

4. A replication-defective retroviral vector according to claim 3, wherein the regulatory sequence is the 578bp element of the WAP promoter-HGH gene hybrid or any other element/region of the WAP regulatory sequence conferring mammary specific expression.

5. A replication-defective retroviral vector according to claim 3, wherein the regulatory sequence is the U3 region of MMTV or subregions thereof conferring mammary specific expression.

6. A packaging cell line preferably of rodent, canine, feline or human origin or a packaging cell line histocompatible with human tissue harbouring:
1) a retroviral vector according to claims 1-5
2) at least one retroviral or recombinant retroviral construct coding for proteins required for said retroviral vector to be packaged.

7. A recombinant vector virus particle obtained by culturing the packaging cell line according to claim 6 under suitable conditions optionally followed by isolation of the recombinant vector virus produced.

8. A pharmaceutical composition comprising the recombinant vector virus particle according to claim 7 or a packaging cell line according to claim 6.

9. A packaging cell line according to claim 6 which is encapsulated in a virus permeable porous membrane.

10. An encapsulated packaging cell line according to claim 9 where the microcapsules are formed by a complex of cellulose sulphate and polydimethyl-diallylammonium.

11. A method for the treatment of breast cancer comprising administering to a human in need thereof a recombinant vector virus particle according to 7 or a packaging cell line according to claim 6 or an encapsulated packaging cell line according to claims 9 or 10.

12. A method for the treatment of pancreatic cancer comprising administering to a human in need thereof a recombinant vector virus particle according to claim 7 or a packaging cell line according to claim 6 an encapsulated packaging cell line according to claims 9 or 10.

13. A retroviral provirus integrated in the human genome carrying a DNA-construct comprising a cytochrome P450 gene under transcriptional control of the carbonic anhydrase II, WAP or MMTV regulatory sequences or an X-ray inducible promoter .

14. A human cell, containing a DNA construct carrying a cytochrome P450 gene or an antisense cytochrome P450 gene under transcriptional control of the carbonic anhydrase II, WAP or MMTV regulatory sequences or an X-ray inducible promoter.

Abstract

A replication-defective retroviral vector carrying a cytochrome P450 gene under transcriptional control of target cell specific regulatory elements or promoters or X-ray inducible promoters.